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April 12, 2007

OUR FILE NO. 20040351.DIB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re App	:	Carol W. Readhead et al.	:	April 12, 2007
S.N.	:	10/008,385	:	Art Unit 1632
Filed	:	November 12, 2001	:	Examiner Joanne Hama
For	:	TRANSFECTION, STORAGE AND TRANSFER OF MALE GERM CELLS FOR GENERATION OF TRANSGENIC SPECIES & GENETIC THERAPIES		

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Transmitted herewith is a Supplemental Paper to Appellant's Brief in response to the Notification of Non-Compliant Appeal Brief dated March 26, 2007 and a Certificate of Facsimile in the above-identified patent application.

The Commissioner is hereby authorized to charge any fees listed in 37 CFR 1.16 and 1.17 which may be required by this paper or credit any overpayment to Deposit Account No. 08-1265.

No additional fee is required.

Yours very truly,

NIKOLAI & MERSEREAU, P.A.

C. G. Mersereau

CGM/acn
Enclosures

CERTIFICATE OF MAILING

I hereby certify that the foregoing SUPPLEMENTAL PAPER TO APPELLANTS BRIEF in triplicate and a Transmittal Letter in application Serial No. 10/008,385, filed November 12, 2001, are being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Mail Stop APPEAL BRIEF - PATENTS, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, postage prepaid, on April 12, 2007.

April C. Nelson
On Behalf of C. G. Mersereau

Date of Signature: April 12, 2007

Our Docket No. 20040351.DIB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

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SUPPLEMENTAL PAPER TO APPELLANTS' BRIEF

Mail Stop APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This paper is submitted in triplicate in response to a Notification of Non-Compliant Appeal Brief dated March 26, 2007. Because the Notice of Non-Compliance involved only a very minor matter, Appellants believe this paper should be sufficient to bring the Brief into compliance.

The only deficiency Appellants find relates to the fact that only the odd numbered pages of the Kim et al 1997 reference were included in the Brief, the even numbered pages being missing.

Accordingly, a complete copy of the Kim et al 1997 reference is attached to this paper.

In addition, reference was made to pages missing before page 54 in the Brief. Appellants do not understand this reference inasmuch as the Brief is concluded on page 23. Therefore, Appellants do not understand the reference to page 54.

It is believed now that Appellants' Brief is in full compliance with the rules and it is requested that there be no more delays and that the Appeal proceed before the Board.

Respectfully submitted,

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MOLECULAR REPRODUCTION AND DEVELOPMENT 46:515–526 (1997)

Development of a Positive Method for Male Stem Cell-Mediated Gene Transfer in Mouse and Pig

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ABSTRACT Classical approaches for producing transgenic livestock require labor-intensive, time-consuming, and expensive methods with low efficiency of transgenic production. A promising approach for producing transgenic animals by using male stem cells was recently reported by Brinster and Zimmermann (1994: Proc Natl Acad Sci 91:11298–11302) and by Brinster and Avarbock (1994: Proc Natl Acad Sci USA 91:11303–11307). However, in order to apply this technique to producing transgenic animals, some difficulties have to be overcome. These include a satisfactory method for short-term in vitro culture for drug selection after transfection with exogenous DNA, and methods for the use of livestock such as pigs. We developed a new method for transferring foreign DNA into male germ cells. Mice and pigs were treated with busulfan, an alkylating agent, to destroy the developing male germ cells, and liposome/bacterial LacZ gene complexes were introduced into each seminiferous tubule by using a microinjection needle. As a control, lipofectin was dissolved in phosphate-buffered saline at a ratio of 1:1, and then injected into seminiferous tubules. In mice, 8.0–14.8% of seminiferous tubule expressed the introduced LacZ gene, and 7–13% of epididymal spermatozoa were confirmed as having foreign DNA by polymerase chain reaction. The liposome-injected testes were all negative for X-gal staining. These results indicate that some spermatozoa were successfully transformed in their early stages by liposome/DNA complexes. In pigs, foreign DNA was also incorporated efficiently into male germ cells, and 15.3–25.1% of the seminiferous tubules containing germ cells expressed the LacZ gene. The data suggest that these techniques can be used as a powerful tool for producing transgenic livestock. *Mol. Reprod. Dev. 46:515–526, 1997.*

Key Words: transgenic; sperm vector; male stem cells; liposome; testis; mouse; pig; LacZ

INTRODUCTION

The first genetically engineered transgenic mice were produced by microinjecting foreign DNA into the pronucleus of a zygote, and then subsequently transferring the zygote into a recipient female (Palmiter et al., 1982; Gordon and Ruddle, 1983; Palmiter and Brinster, 1986; Brinster et al., 1993). Although a number of researchers have reported production of transgenic sheep (Hammer et al., 1985; Wright et al., 1991), pigs (Pursel et al., 1989), and cattle (Bowen et al., 1994; Strijker et al., 1992), progress with livestock has been hampered for several reasons. A fundamental problem in the production of transgenic livestock is a limited supply of early embryos at the proper stages of development. Furthermore, it is very expensive and laborious to obtain zygotes by superovulation and surgical collection, and this often results in an asynchronous population of embryos. In addition, both the survival of a zygote after microinjection and the integration frequency of exogenous genes in farm animals are very low (Eyestone, 1994). Therefore, the sperm vector as an alternative methodology has been developed (Arezzo, 1989; Atkinson et al., 1991; Lavitrano et al., 1989, 1992; Laura and Gandolfi, 1993; Zani et al., 1995), but this technique remains unproved and is still under examination (Al-Shawi et al., 1990; Brinster et al., 1989; Maddox, 1989). Even though several theoretical approaches, such as retroviral vectors (Kim et al., 1993) and embryonic stem cells (Anderson, 1992; Stewart, 1991), have been addressed, no group has produced transgenic livestock by using these methods.

Recently, it was reported that early male germ cells, before the onset of chromatin condensation, were successfully transfected and cultured in vitro to round spermatids having haploid chromosomes (Hoffman et al., 1992, 1994; Minoo et al., 1993; Plauis-Flucklinger et al., 1993). Brinster and Avarbock (1994a) and Brinster and Zimmermann (1994b) reported a technique with which they transplanted testis-derived cells into mouse seminiferous tubules of infertile recipients and produced progeny derived from donor male germ cells. However, the application of these two techniques to produce transgenic animals will depend upon the cul-

Abbreviations: X-gal, 5-brome-4-chloro-3-indolyl-D-galactoside; PCR, polymerase chain reaction; FBS, fetal bovine serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; IVF, in vitro fertilization; PMSG, pregnant mare's serum gonadotropin; hCG, human chorionic gonadotropin; EDTA, ethylenediaminetetraacetic acid.

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Received 16 May 1996; Accepted 10 October 1996

ture conditions of undifferentiated spermatogonia during gene manipulation in vitro. In addition, the seminiferous tubules of a pig, unlike those of a mouse, are hardly visible under light microscopy.

Mammalian spermatogenesis involves a highly regulated proliferation and differentiation of germ cells within the seminiferous epithelium. In the production of transgenic animals, potentially, transfection of testicular stem cells might be used with advantage, as they undergo self-renewal and replicate large numbers of pluripotent stem cells, while differentiated spermatogonia produce only four spermatozoa. For this purpose, developing male germ cells destroyed by treatment with busulfan (Bucci and Meistrich, 1987) might be replaced by male germ cells transfected directly by introducing liposome/DNA complexes into mouse seminiferous tubules or pig testes. In this study, we asked whether direct transfection of liposome/DNA complexes into the germ cell-depleted testes with only male stem cells could increase the efficiency of production of transgenic livestock. The rationale is based on the assumption that transfected male stem cells should replicate and/or differentiate into mature sperm cells, which might transfer foreign DNA into the zygote genome at fertilization.

MATERIALS AND METHODS

Mice

Young ICR mice (30–40 g, 8–12 weeks old) were housed in wire cages and fed ad libitium. The mice were maintained at 22 ± 1°C under a 12-hr light-dark cycle with 70% humidity. Animals were maintained and experiments were conducted in accordance with the Kon-Kuk University Guide for the Care and Use of Laboratory Animals.

Preparation of Spermatozoa

Epididymal spermatozoa were obtained from caudal epididymides of ICR mice as described by Lavitrano et al. (1989). Sperm was adjusted to a final concentration of 3×10^6/ml by hemocytometer counting and incubated for 15 min at 37°C in 5% CO_2 in air for transfection with foreign DNA.

Transfection of Spermatozoa

The DNA (5.89 kb) derived from the pZIP(X)hEPOSV-neo plasmid (Goto et al., 1988), which contains human erythropoietin and neo genes digested with *Hind*III and *Eco*RI, was used for in vitro transfection of spermatozoa. The DNA (0.6 μg) and liposome (2 μg, Gibco) were separately diluted in 20 μl of sterile water and allowed to stand for 30 min at room temperature, followed by gentle mixing of both solutions. Spermatozoa were washed twice with 1 ml of bovine serum albumin (BSA)-free in vitro fertilization (IVF) medium (Lavitrano et al., 1989). Spermatozoa at 1×10^6 cells/ml were transfected with both linear DNA derived from pZIP(X)-hEPOSVneo plasmid and liposome in BSA-free IVF medium for 2 hr at 37°C, 5% CO_2 in air. The sperm cells were incubated further for 1 hr after adding BSA (10 mg/ml) to induce capacitation. The transfected spermatozoa were thoroughly washed with phosphate-buffered saline (PBS) containing 10% fetal bovine serum (FBS), and then analyzed by flow cytometry and in situ hybridization. To examine DNA uptake in spermatozoa, spermatozoa were then treated with DNase I to remove exogenous DNA bound onto spermatozoa.

In Vitro Fertilization

Female mice were superovulated by sequential injection of 5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma), followed 48 hr later by 5 IU of human chorionic gonadotropin (hCG, Sigma). Oocytes were recovered from ampullae at 12–15 hr post-hCG in PBS containing 10% FBS. In vitro fertilization was performed by placing approximately 30 oocytes in 50-μl droplets of IVF medium (Lavitrano et al., 1989), and 2 μl of the transfected sperm suspension (final concentration in droplet, 1×10^6 spermatozoa/ml) were added.

Flow Cytometry

DNA contents of control and treated spermatozoa, both with and without DNase I digestion following transfection with foreign DNA, were estimated by flow cytometry (FACStar, Beckon Dickinson) after staining with propidium iodide (1 μg/ml). Cell numbers were represented by intensity of staining. Chicken erythrocytes were used as an internal standard for flow cytometry. The DNA content in spermatozoa was determined at 460 laser light (ultraviolet).

In Situ Hybridization

The transfected sperm samples were fixed with 4% paraformaldehyde for 15 min, smeared on gelatin-coated glass slides, treated with 0.2 N HCl and 1 μg/ml of proteinase K at 37°C for 30 min, and then refixed with 4% paraformaldehyde according to a slightly modified method described previously (Bachiller et al., 1991; Camaioni et al., 1992; Francolini et al., 1993). After washing in PBS for 15 min at room temperature (RT), hybridization buffer containing 20 ng of probe DNA, 6 × SSC, 5 × Denhardt's solution, 45% formamide, and 10% dextran sulfate was placed on each slide and heated for 5 min at 95–100°C. Samples were briefly chilled on ice and incubated for 4 hr at 42°C. After incubation, slides were rinsed twice in 4% formamide and 6 × SSC for 15 min at 42°C, and then washed twice in 2 × SSC for 5 min at RT and once in 0.2 × SSC for 15 min at 50°C. The signal of the exogenous DNA in spermatozoa was detected as a green fluorescence of the FLUOS-conjugated anti-digoxigenin antibody by epifluorescence microscopy. Sperm nuclei were counterstained with propidium iodide at a concentration of 0.5 μg/ml.

Analysis of Integration Patterns

To avoid the ligation of linear DNA, such as head and tail array after in vivo transfection, the DNA was strictly treated with calf intestine alkaline phosphatase

(CIAP, Takara) and tested by ligation reaction. Spermatozoa transfected with a 5.89-kb *Hind*III/*Eco*RI fragment derived from pZIP(X)hEPOSVneo plasmid were investigated for an array of integrations by PCR. Primers for PCR were prepared according to the method proposed by Burdon and Wall (1992): for detection of head and tail (340 bp), anti 5'-CGTAAACTGAACCAAGATACG-3' (primer 1), and 5'-CAGGAGTGGGGAGGCACGAT-3' (primer 2); for detection of single copy (867 bp), 5'-GAGTTGGGAAGCTAGACACTG-3', and anti 5'-CTCTCCCTCCTGCCCCTCAGCAGC-3'. PCR of spermatozoa was performed according to the method described by Li et al. (1988): the spermatozoa were treated with DNase I, and then completely washed, boiled at 95°C for 10 min to extract DNA, and cooled rapidly for 3 min. At 15 hr after in vitro fertilization, zygotes were enucleated by drawing the male pronucleus into a 20-μm beveled pipet as described by Hogan et al. (1994). After enucleation, a male pronucleus was washed into the oocyte manipulation medium, boiled in PCR buffer at 100°C for 5 min, and analyzed by PCR as described above.

Busulfan Treatment

Mice and pigs were treated with busulfan, an alkylating agent, according to the previous methods with minor modifications (Bucci and Meistrich, 1987; Brinster and Zimmermann, 1994b). Busulfan (40–100 mg/kg of body weight) was completely dissolved using 50 μl of N, N-dimethy-formamide (Sigma), and then 50 μl of sesame oil were added. The solution was injected intraperitoneally once in mice or twice every week for 5 weeks in pigs. In this study, 28 mice and 8 pigs from age 8–12 weeks were used. Among them, 19 mice and 4 pigs were used for histological analysis following busulfan treatment. Nine mice and 4 pigs were used for gene transfer.

Transfer of Liposome/DNA Complex Into Seminiferous Tubules

The mouse testis was exposed by laparotomy. The tunica albuginea of both testes was pierced partially by using a sharp 30-gauge needle, and approximately 5–10 μl of liposome/bacterial LacZ gene, derived from pCH110 plasmid under the control of SV 40 promoter (Takeda and Toyoda, 1991), complexes (4 μg/1 μg) were introduced into a site of seminiferous using a microinjection needle under light microscopy (Fig. 3). In the pigs, 4 weeks after the first busulfan treatment, the testes were exposed by surgical operation. Then, approximately 500 μl of DNA/liposome (rate of 1:4; 10 μg/40 μg) complexes were randomly introduced into 3 or 4 sites of both testes using a glass microinjection needle after piercing the tunica albuginea with a 30-gauge needle. Mice and pigs were injected with the liposome/DNA complexes twice 7 days between treatments.

Separation of Male Germ Cells

Spermatogenic and spermiogenic cells were prepared from the testes and purified by the Celsep® (Eppendorf) separation system, according to the method described by Bellve (1993) with minor modifications. Briefly, 4–8 weeks after transfection, the tunica was removed and testes were dissociated by mincing with scissors. The cells were incubated for 30 min at 37°C in HBSS (58.44 mM NaCl, 74.55 mM KCl, 137.9 mM Na_2PO_4, 0.1% glucose, and 84.01 mM $NaHCO_3$) containing 1 mg/ml of collagenase type I (Sigma, catalogue no. C-0130) and 1 μg/ml of DNase I. After centrifugation, cells were resuspended with 0.25% trypsin/1 mM EDTA and incubated for 5 min at room temperature. Then, trypsin was removed by centrifugation, and cells were separated on a linear gradient of 2–4% Percoll in PBS. After 2 hr of sedimentation at 4°C, cells were collected as 40-ml fractions. The cell types of each fraction were examined under light microscopy, and fractions containing the same cell types were pooled, washed twice with PBS, and then analyzed for LacZ expression.

X-Gal Assay

Four to 8 weeks after transfection, testes were dissociated and fixed in 2% formaldehyde for at least 2 hr, and then stained overnight in solution (1.0 mg/ml of X-gal, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS, (pH 7.4) (Vernet et al., 1993). The stained testes were fixed again, embedded in paraffin, and sectioned by microtome. Expression of the bacterial LacZ gene in testes was investigated under light microscopy. Spermatocytes and round/elongating spermatids separated by Celsep® or preimplantation embryos were fixed on fixation solution (2% formaldehyde and 0.2% glutaraldehyde in PBS, pH 7.4, without Mg^{+2} and Ca^{+2} for 10 min at 4°C). The germ cells were washed twice in PBS, and stained with X-gal for 12 hr.

Immunohistochemistry

Antibody against β-galactosidase was purchased from Oncogene Science. Tissue for immunostaining was cleared in histoclear for approximately 10 min and dehydrated in decreasing concentrations of ethanol. Immunohistochemistry was performed according to standard procedures provided by the manufacturer (Mouse, Rabbit and Rat UniTect® Immunohistochemistry System, Oncogene Science). Sections were placed in 3% peroxide in pure methanol and 0.1% of pepsin in 0.05 N HCl, pH 2.25, for 30 min to reduce background staining. Sections were washed twice (5 min each) in TBS (0.05 M Tris-HCl, pH 7.4, and 0.85% NaCl) and blocked with normal horse serum diluted in TBS (1:5; NSS-TBS). Sections were incubated for 30 min with primary β-galactosidase antibody diluted at a concentration of 1:500 in NHS:TBS. One drop of horse serum from the ABC Kit was used as a negative control. Excess antibody was removed by washing twice for 5 min with TBS, and then biotinylated secondary IgG was added for 30 min, with a rinsing with 3 changes of TBS for 5 min. Sections were incubated with ABC reagent for 30 min and washed extensively with TBS, and rinsed in 1% Triton-X-PBS for 30 sec. The color

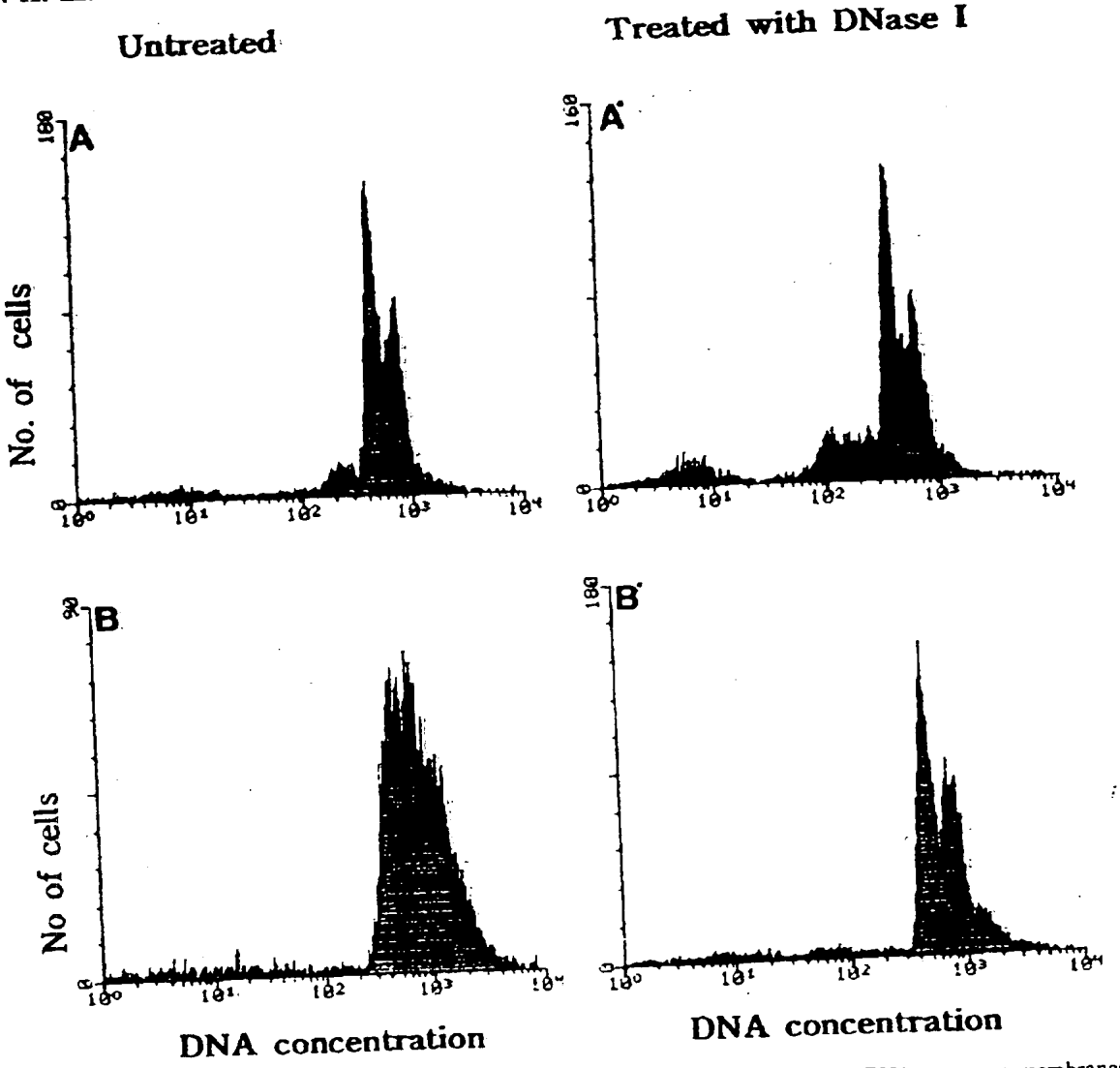

Fig. 1. Analysis of DNA concentration in spermatozoa transfected with foreign DNA by flow cytometry. DNA contents were determined at 460 laser light (ultraviolet) to examine binding or uptake of foreign DNA on transfected spermatozoa. After transfection, spermatozoa were thoroughly washed to remove foreign DNA which had not interacted with spermatozoa. DNase I treatment was used to remove simple binding of exogenous DNA on sperm membranes. Data are depicted by a histogram in which the relative number of cells appears on the y axis and the intensity of fluorescence in arbitrary units appear on the x-axis. A and A': Control spermatozoa. B: Spermatozoa transfected with exogenous DNA. B:' Spermatozoa treated with DNase I following transfection with foreign DNA.

reaction was developed with a solution of 0.5% diaminobenzidine in 0.05 M Tris-HCl, pH 7.6, containing 0.01% hydrogen peroxide. After development of the color reaction, sections were washed in water, dehydrated, and mounted with a coverslip.

RESULTS

Transfection of Spermatozoa

To examine the interaction of liposome/DNA complexes with epididymal spermatozoa in vitro, spermatozoa transfected with liposome/DNA complexes were analyzed by using flow cytometry and in situ hybridization. Figure 1 shows the binding of exogenous DNA on spermatozoa by flow cytometry, indicating that the liposome/DNA complexes were efficiently transfected onto/into spermatozoa. The binding of foreign DNA onto spermatozoa was confirmed by in situ hybridization (Fig. 2). Spermatozoa transfected with liposome/DNA complexes (64/100) showed a high frequency of binding of exogenous DNA. Furthermore, the binding onto spermatozoa of exogenous DNA was resistant to DNase I treatment, which indicated the presence of exogenous DNA inside the sperm membrane. However, expression of DNA transfected into spermatozoa was not found in embryos after fertilization in vitro (data not shown). Thus, male pronuclei removed from oocytes fertilized

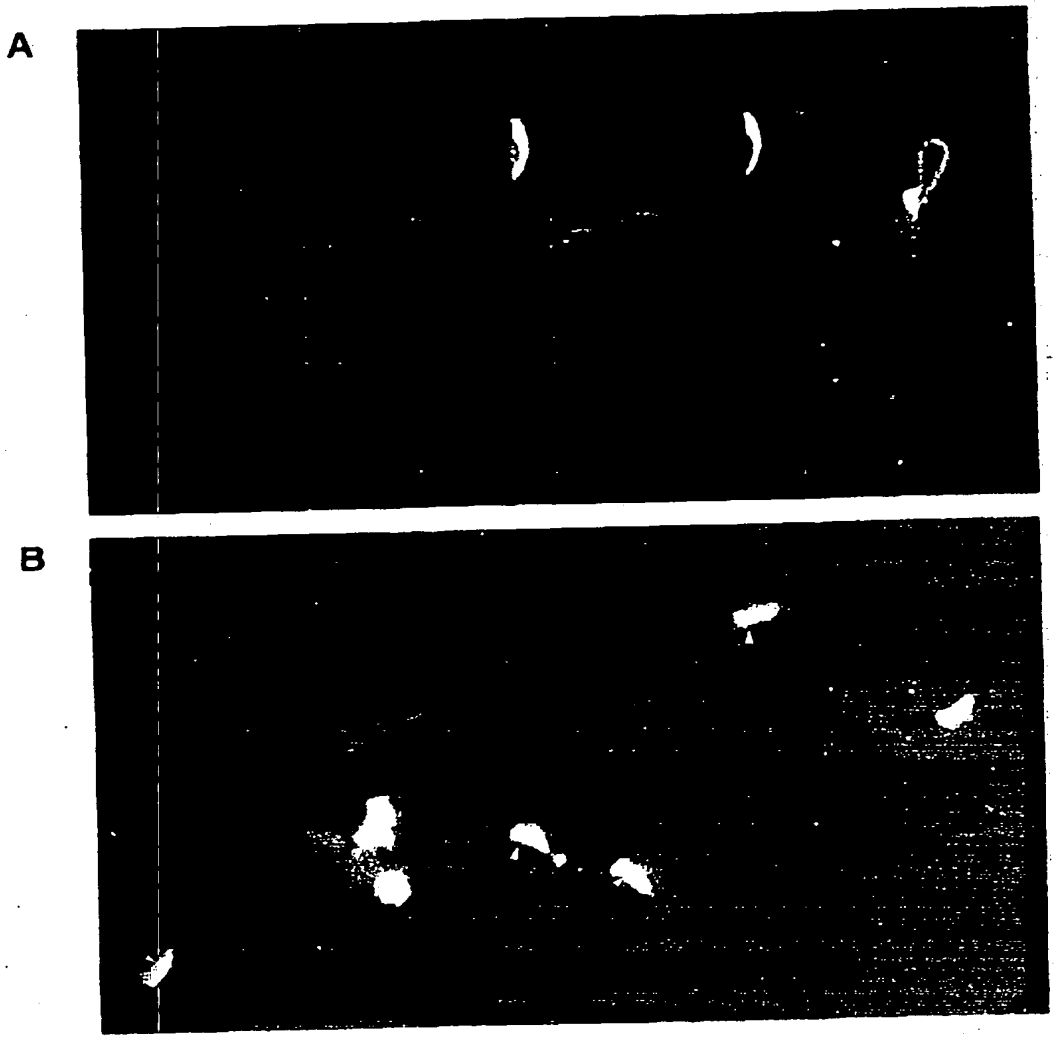

Fig. 2. Association of exogenous DNA in spermatozoa examined by fluorescent in situ hybridization. The signal of the exogenous DNA was detected as a green fluorescence of the FLUOS-conjugated anti-digoxigenin antibody by epifluorescence microscopy. A and B: Patterns of exogenous DNA binding in sperm cells. Yellow represents a complementary color between green and red. Yellow or green (arrow) indicates sperm cells labeled with foreign DNA.

by the transfected spermatozoa were examined for the presence of exogenous DNA. By PCR, exogenous DNA was not detected in enucleated nuclei (0/20), but was occasionally detected in the cytoplasm (2/30). These data indicate that liposome/DNA complexes can be bound onto spermatozoa efficiently, but cannot be incorporated into their chromosome DNA.

In Vivo Transfection of Male Stem Cells

Using sections of mouse testes, we investigated the effects of busulfan in the destruction of developing spermatocytes after treatment with busulfan. At 4 weeks after treatment with busulfan, only type A spermatogonia and a few developing spermatocytes in seminiferous tubules remained. At 12 weeks, the remaining stem cells were actively dividing and they showed a similar spermiogenesis and spermatogenesis (data not shown). To obtain efficient stem-cell transfection, male mice were pretreated with busulfan. Three weeks after injection with busulfan, liposome/DNA complexes were directly transferred into mouse seminiferous tubules (Fig. 3). Five of the 9 mice lost body weight severely and died within 2 weeks of transfer. However, testes from both dead and living mice efficiently expressed the bacterial LacZ gene in spermatogonia and developing spermatocyte cells, as well as in Leydig and Sertoli cells (Figs. 4, 5). Furthermore, the bacterial LacZ gene was broadly expressed in spite of partial transfer of foreign DNA into the seminiferous tubules. Control testes transfected with DNA-free liposomes did not show staining at any of the stages examined. To prove expression of exogenous β-galactosidase, expression of β-galactosidase was also confirmed by immunohistochemistry analysis using

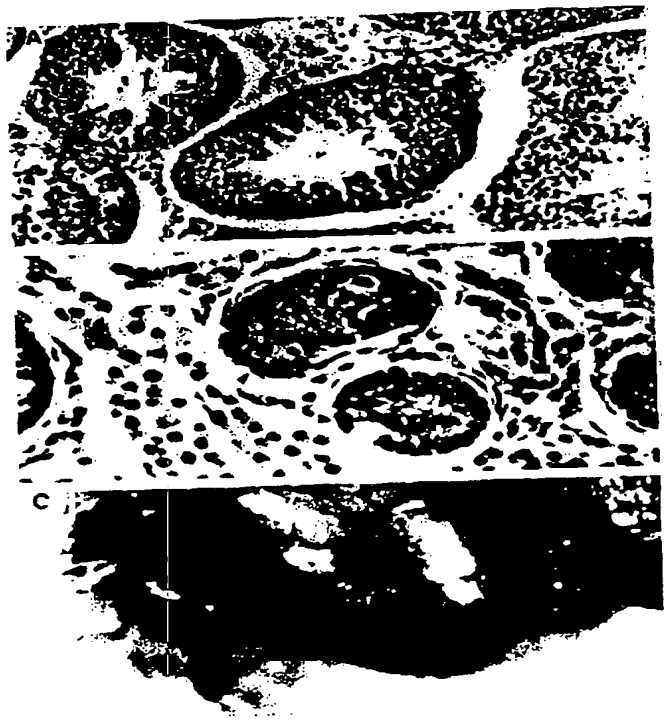

Fig. 3. Transfer of liposome/DNA complexes into mouse seminiferous tubules. For DNA transfection, see Materials and Methods. A: Control seminiferous tubules (×400). B: Light microscopy of seminiferous tubule cross sections at 3 weeks after treatment with busulfan (×400). At 3 weeks after busulfan administration, most germ cells had degenerated, and only type A spermatogonia and some spermatocytes remained. Arrow, spermatogonia. C: Tunica albuginea was pierced partially by using a sharp 30-gauge needle, and then liposome/DNA complexes were introduced into the seminiferous tubule by using a micrninjection needle under a microscope (×40). Arrow, exposed seminiferous tubules.

β-galactosidase antibody (Fig. 5). One week after transfection, the signal was restricted to only type A spermatogonia. However, 6 and 8 weeks after introduction of the bacterial LacZ gene, the signal was extended to developing spermatocytes, indicating that liposome/DNA complexes transfected male stem cells efficiently. Furthermore, the introduced DNA existed in male germ cells over 1 month and also showed head and tail array, indicating that some exogenous DNA was integrated into the genome (Table 1). To examine the stable expression of the LacZ gene 7 weeks after transfection, spermatocytes and round/elongating spermatids separated by Celsep® were analyzed by X-gal staining. Approximately 3.7–10.3% of round and elongating spermatids in each testis showed positive X-gal staining (Table 2). The total number of germ cells in both busulfan-treated and control groups were approximately 5×10^6 and 3×10^8, respectively. Twelve weeks after transfection in busulfan-treated mice, even though the number (1×10^4) of spermatozoa recovered from caudal epididymides was low, 7–13% of epididymal spermatozoa showed the presence of the introduced

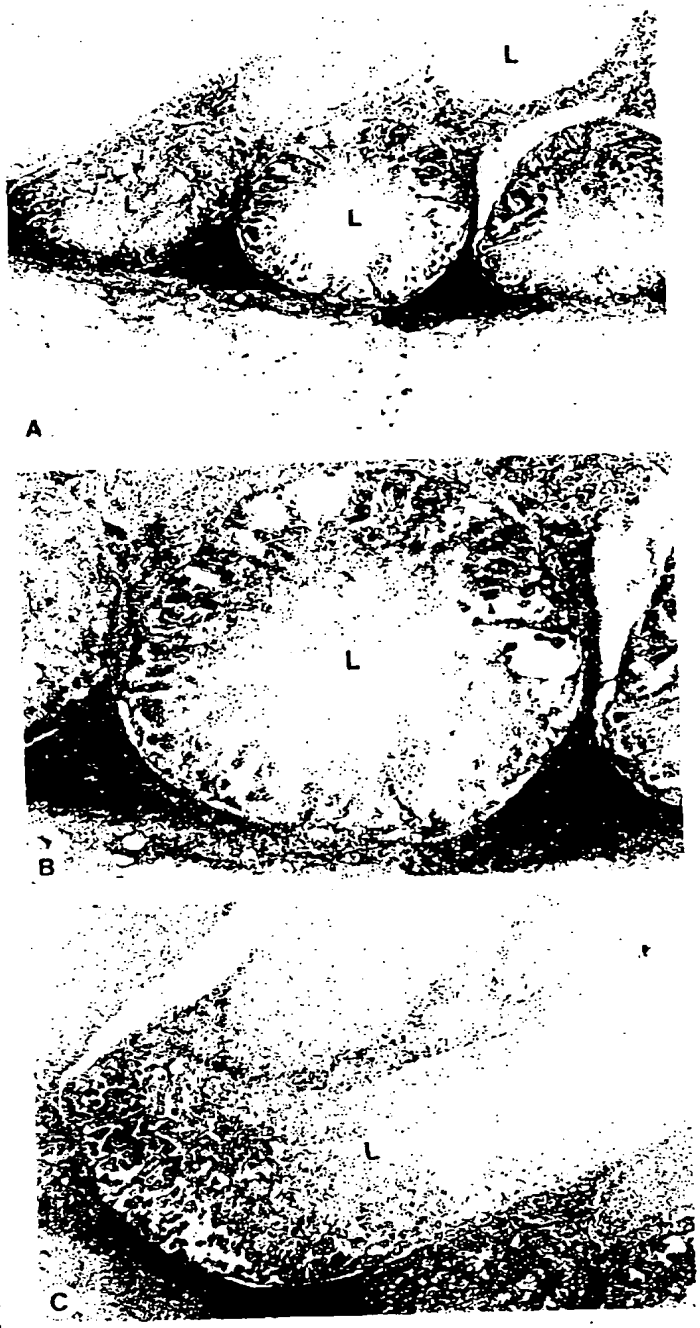

Fig. 4. Transient and stable expression of LacZ gene in mouse male germ cells. For X-gal staining, see Materials and Methods. Bacterial LacZ gene was expressed in spermatogonia and some spermatocytes (×100 and ×400, A and B) and also in developing spermatocytes and round spermatids (×400, C). L, seminiferous tubule. Arrows in B and C, developing male germ cells.

DNA by PCR analysis (Table 2). Although the number of sperm cells containing foreign DNA in the ejaculated sperm was low, the result indicates that the in vivo transfection system could be used as a potential vector for production of transgenic animals. In the pig, unlike

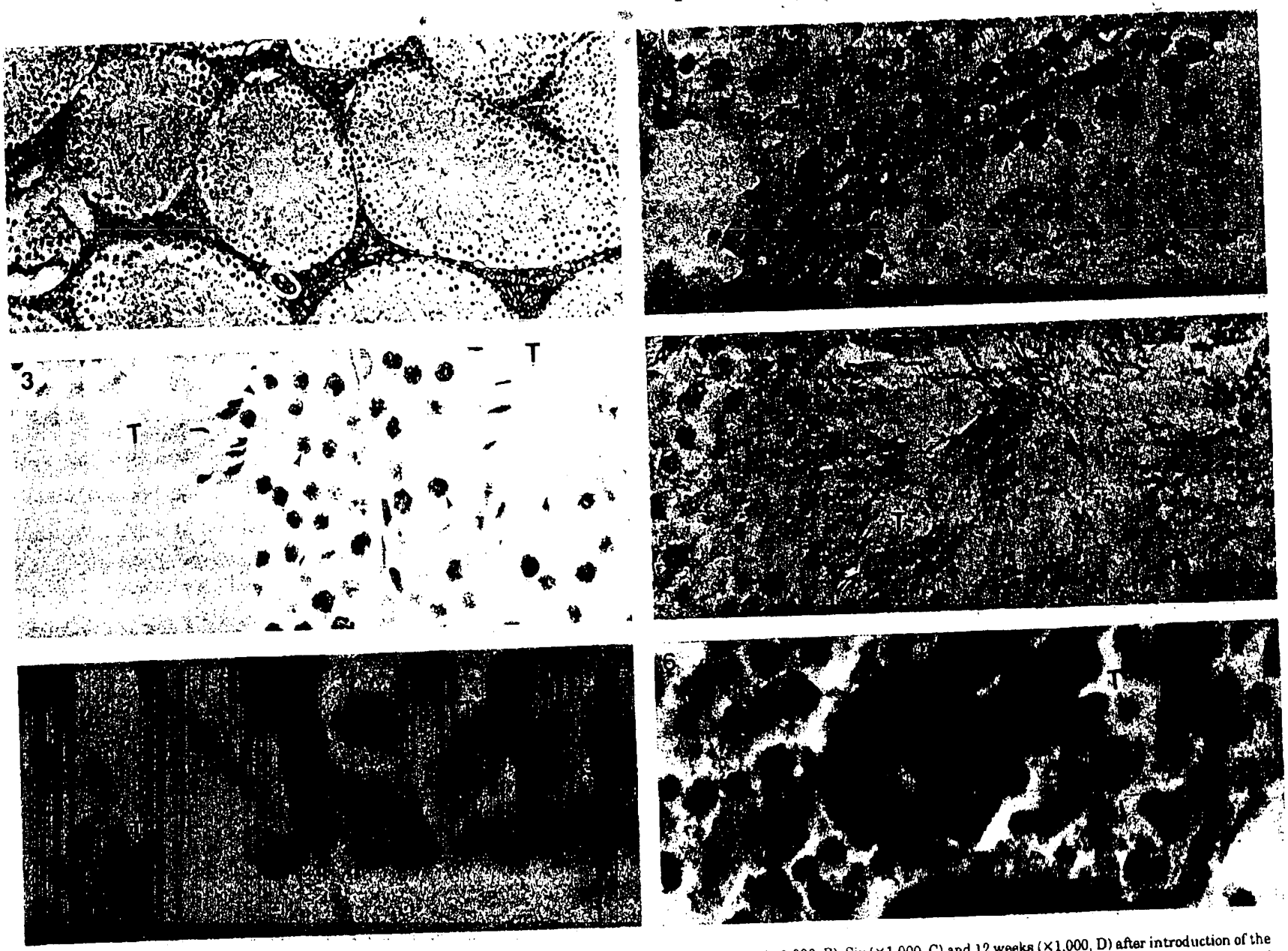

Fig. 5. Immunohistochemistry localization of bacterial LacZ gene in transfected testis. For immunocytochemistry, see Materials and Methods. Testes in different stages of development following busulfan and gene transfer were fixed and labeled by immunohistochemistry with β-galactosidase antibody (1–4) and by X-gal staining (5, 6). After transfection with the bacterial lacZ gene, most of the signal was restricted to Leydig cells (×100, A) and type A spermatogonia (×1,000, B). Six (×1,000, C) and 12 weeks (×1,000, D) after introduction of the lacZ gene, the signal extended to developing spermatocytes and mature spermatozoa. By X-gal staining, some type A spermatogonia partially expressed the lacZ gene (×1,000, E), and some germ cells showed whole staining (×1,000, F). Arrowhead, male germ cells expressing the bacterial LacZ gene.

TABLE 1. Expression Rate and Integration Pattern of Exogenous DNA in Testis Following Introduction of Liposome/DNA Complexes

Species	No. of tubules expressed (%)/investigated[a]	Integration pattern[b]	Conditions
Mouse 1	4/173 (2.3)	Head/tail	Dead
Mouse 2	7/146 (4.7)	Head/tail	Dead
Mouse 3	21/143 (14.6)	Head/tail	Dead
Mouse 4	16/143 (11.1)	Head/tail	Dead
Mouse 5	25/194 (12.8)	Head/tail	Dead
Mouse 6			Alive
Mouse 7	19/160 (11.8)	Head/tail	Alive
Mouse 8	22/174 (12.6)	Head/tail	Alive
Mouse 9		Single band	Alive
Pig 1	72/340 (21.1)	Head/tail	Alive
Pig 2	54/321 (16.8)	Head/tail	Alive
Pig 3	47/187 (25.1)		Dead
Pig 4	69/450 (15.3)	Head/tail	Dead
Pig 5			Control

[a]Expression of LacZ gene in testis sections was examined by X-gal staining.
[b]Integration pattern of introduced foreign DNA was analyzed by PCR.

TABLE 2. Evaluation of LacZ Expression in Male Germ Cells Purified by Celsep™ Method at 57 Days After Transfection With Liposome/DNA Complexes

Mouse	No. (%) of X-gal-positive cells/no. of spermatogenic cells	No. (%) of X-gal-positive cells/no. of spermiogenic cells	No. (%) of PCR-positive sperm/no. of epididymal sperm
1	48/259 (18.3)	37/358 (10.3)	13/100 (13)
2	4/658 (2.1)	16/268 (5.9)	11/100 (11)
3	76/348 (21.8)	34/896 (3.7)	7/100 (7)

the mouse, it was impossible to distinguish a seminiferous tubule by microscopic observation because the testis of the pig is surrounded by a thick and opaque tunica albuginea. Thus, we slightly modified the technique for the pig. Eight weeks after transfection, pig testes were analyzed for expression of the LacZ gene. As shown in Figure 6, in the testes of the pig, bacterial LacZ gene/liposome complexes were selectively absorbed within seminiferous tubules, whereas the lacZ gene was clearly expressed in the seminiferous tubules containing male germ cells. The rates of LacZ expression in testes sections of mice and pigs were 8.0–14.8% and 15.3–25.1% of total tubules, respectively. This result was compatible with that of mouse spermatocytes and round/elongated spermatids (Tables 1, 2).

DISCUSSION

Our aim was to establish whether sperm cells could be used as a potential vector for producing transgenic animals. The present studies have shown that liposome/DNA complexes efficiently transfected male germ cells in vivo. In the mouse, expression of DNA introduced into the seminiferous tubules was found in male stem cells including developing germ cells and epididymal spermatozoa. In the pig, DNA complexes, in this case also injected directly into the testis, induced expression of foreign DNA in male germ cells. Transfected stem cells may therefore be utilized as a tool for the production of transgenic animals, while transfection of epididymal sperm cells could not be accomplished.

To utilize spermatozoa as a vector for gene transfer, we focused on the possibility of producing transgenic animals by in vivo transfection of male stem cells. Male germ cells are divided to produce spermatogonia with two main types, noncycling (A^0) and those that differentiate into spermatocytes and produce only four spermatozoa (Bellve, 1993; Bucci and Meistrich, 1987). Therefore, it is very important to obtain efficient transfection of undifferentiated noncycling spermatogonia in synchronized testes for producing transgenic animals. However, busulfan, which was used to obtain germ cell-depleted testes with only male stem cells, was very toxic and killed a high percentage of mice. On the other hand, animals fed a diet lacking vitamin A showed a high survival rate (unpublished data). Also, lethally treated mice can be protected by single or combination injections with cytokine, such as interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), granulocyte/macrophage colony-stimulating factor (G-CSF), and stem cell factor (C-kit), or by transplantation of normal bone marrow (Zsebo et al., 1996).

Three weeks after treatment with busulfan, liposome/DNA complexes were introduced into each seminiferous tubule by using a microinjection needle under the light microscope. All of the testes enucleated from dead and living mice expressed the bacterial LacZ gene in the developing spermatocytes and showed stabilized integration into the genome (Table 1). Seven weeks after transfection, expression of exogenous DNA in spermatogonia or spermatocytes suggested that foreign DNA was integrated into the undifferentiated spermatogonia (Figs. 4, 5). Apart from the partial transfer into a seminiferous tubule, the most striking result of these experiments was that the bacterial LacZ gene was broadly expressed. This suggested that some liposome/DNA complexes flowed with fluid into the seminiferous tubule, and that germ cells or somatic cells were transfected by the complexes flowed into another area.

To determine the percentage of blue vs. nonblue rates for expression of the LacZ gene in the spermatid reproduced from male stem cells 7 weeks after transfection, spermatocytes and round/elongated spermatids separated by Celsep™ were stained with X-gal and counted on a hemocytometer. By X-gal staining, approximately 3.7–10.3% of round and elongated spermatids in each testis expressed exogenous DNA (Table 2), although this was never seen in testes transfected with

Fig. 6. Stable expression in porcine male germ cells. Pigs were treated with busulfan (40–100 mg/kg of body weight), as described in Materials and Methods. A: Lumen containing male germ cells (×100). B: Lumen containing male germ cells (×400). C: Peritubular cells (×100). D: Leydig cells in seminiferous tubules (×200). Blue (arrow) represents LacZ expression.

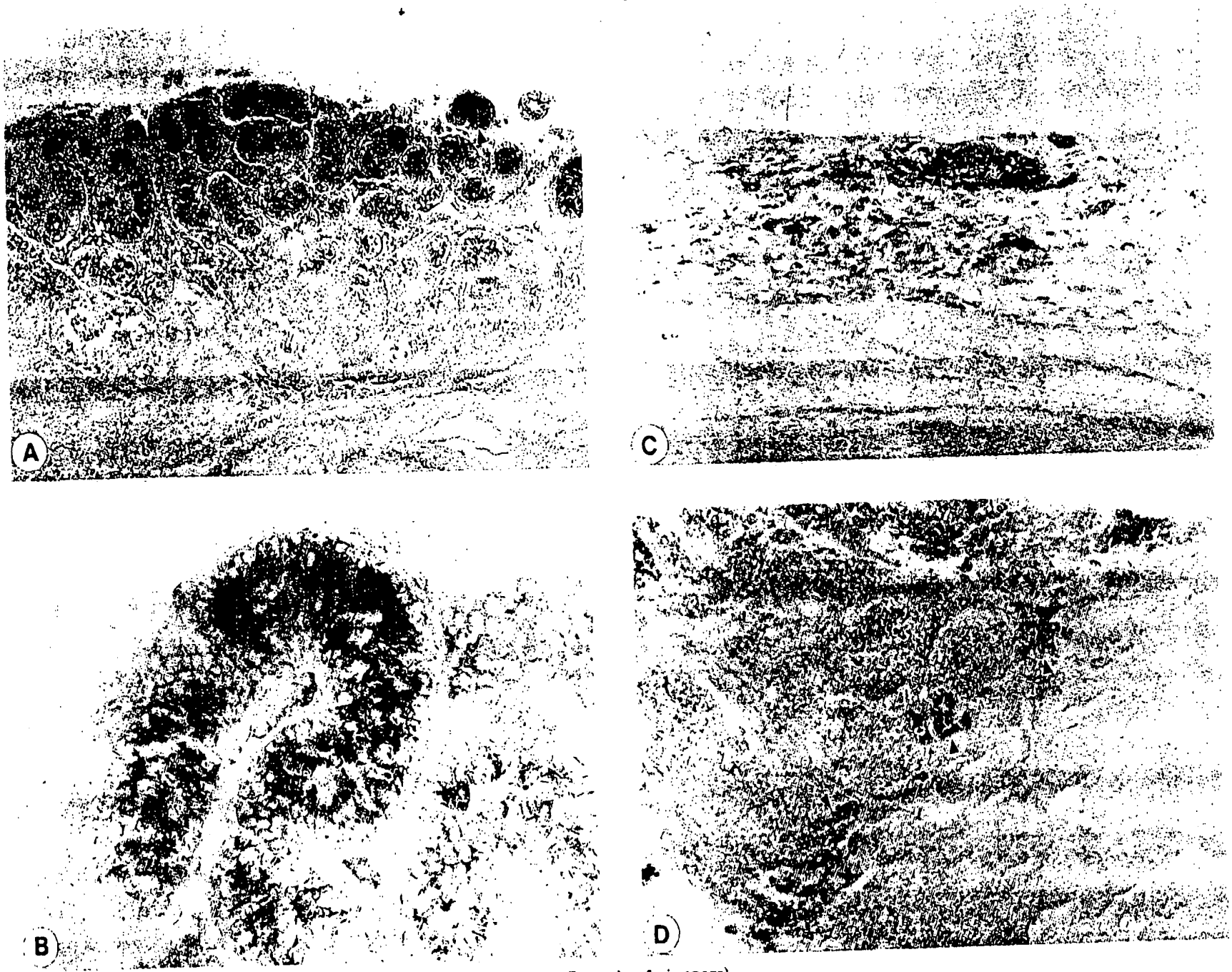

Fig. 6. (Legend on facing page).

DNA-free liposomes. However, the total number of germ cells in both busulfan-treated and control groups was approximately 5×10^6 and 3×10^8, respectively. Also, the number of spermatozoa in busulfan-treated mice (1×10^4) compared to that of control mice was very low. The viability of spermatozoa was also very low, but 7–13% of epididymal spermatozoa were confirmed as having the transfected DNA by PCR (Table 2). This finding suggested that liposome/DNA complexes successfully transformed early germ cells and indicated that the foreign DNA was integrated into the host chromosome of spermatozoa.

Since the seminiferous tubule of pigs is surrounded by a thick and opaque tunica albuginea, application of this technique to the pig required a slight modification. Even though liposome/DNA complexes were introduced randomly into the testis, bacterial LacZ gene/liposome complexes were selectively absorbed within seminiferous tubules, and the LacZ gene was clearly expressed in the seminiferous tubules containing male germ cells (Fig. 6). The optimal dose of busulfan has not been determined; pigs pretreated once with a large dose of busulfan (>200 mg/kg of body weight) died after 3–6 weeks. The cause of death might have been disordered blood production, because busulfan also kills hematopoietic stem cells (Bucci and Meistrich, 1987).

The other approach, recently applied to mouse male germ cells in vitro, is the use of SV40 T antigen to establish immortalized cell lines. It was reported that differentiated male germ cells, before the onset of chromatin condensation, were successfully transfected and cultured in vitro to round spermatids having haploid chromosomes (Hoffman et al., 1992, 1994). It was also reported that the mouse mature oocyte can be fertilized by fusion with a round spermatid isolated from seminiferous tubules, and that the embryo is capable of development to term (Ogura et al., 1993, 1994). The efficiency of producing transgenic livestock may be improved by applying these techniques, while the efficiency for in vitro culture of differentiating or differentiated male germ cells will be improved (Felgner et al., 1989; Rottman et al., 1992).

We examined the possibility of using sperm cells as a vector for transferring exogenous DNA. Since lipofectin cationic liposome can spontaneously interact with RNA/DNA and the complexes can also associate with negatively charged cell membranes such as a sperm head (Bachiller et al., 1991; Francolini et al., 1993; Stewart, 1991), we transfected mature spermatozoa with liposome/DNA complexes. As shown in Figure 1, total DNA content in transfected spermatozoa was increased and was still resistant to DNase I. This result suggests that spermatozoa were at least bound to, or taken up by, the exogenous gene. The taking up or binding of DNA by spermatozoa has been suggested to increase the transportation of foreign DNA into zygote cytoplasm or nuclei fertilized with the transfected spermatozoa (Francolini et al., 1993). Therefore, it was important to characterize whether or not liposome/DNA complexes were internalized into sperm membranes. If the exogenous DNA associated with spermatozoa was able to penetrate through the barriers of the sperm membrane and/or to integrate into sperm chromatin, foreign DNA would naturally participate as a component of the male pronucleus by in vitro fertilization. By contrast, if DNA was not internalized in the sperm nucleus, simple binding of exogenous DNA onto spermatozoa would be ultimately diluted and would disappear in developing embryos (Laura and Gandolfi, 1993). As shown in Figure 2, in situ hybridization studies demonstrate that exogenous DNA was located on the sperm membrane or inner membrane. Even though fluorescence in situ or autoradiography provides an alternative analysis for DNA binding or uptake of foreign DNA onto spermatozoa, this methodology seems to be impractical for proving internalization of exogenous DNA into the sperm nucleus. Since in situ hybridization requires treatment of sperm with a hyper/hypo-osmolar solution, it is possible that exogenous DNA bound onto sperm cells can penetrate into the sperm membrane. Therefore, Francolini et al. (1993) showed that exogenous DNA was internalized or taken up into sperm cells, but Camaioni et al. (1992) did not report whether labeling patterns of spermatozoa by exogenous DNA were intracellular or on the membrane. To determine if sperm cells simply bound, or internalized the exogenous DNA, we investigated the presence of multicopies of plasmid DNA, which is the indicator of integration into the chromosome. By analysis of PCR products, we could not detect the presence of multicopies in spermatozoa and only detected a single band, which indicates only membrane binding of exogenous DNA onto sperm cells (data not shown). Therefore, it is questionable whether sperm transferred foreign DNA into the oocyte nuclei. Considering that the packaging of sperm DNA, unlike somatic cells, was highly condensed into a small volume by protamine (Cotten and Chalkley, 1985; Ward and Coffey, 1989, 1992; Wright et al., 1991; Zani et al., 1995), it can be surmised that exogenous DNA will not integrate into the sperm nucleus but will simply bind to the sperm membrane. Thus, our results lead us to conclude that the introduction of exogenous DNA into mature spermatozoa may be an impractical technique for producing transgenic animals.

In this study, we found that exogenous DNA can be efficiently incorporated and expressed in male germ cells by direct introduction of liposome/DNA complexes into the male testis, in both mouse and pig. This indicates that this technique can be utilized as an efficient approach for producing transgenic animals. However, at the moment we have no representative data as regards how many transgene-positive male germ cells represent truly transgenic fetuses. So far we have examined the possibility of transfections of exogenous DNA at the level of male germ cells. In addition, we have obtained 0–65% of X-gal-positive preimplantation embryos by this method (unpublished data). However, it may be necessary to improve the transfection efficiency of male germ cells because the number of spermatozoa with exogenous DNA among spermatozoa

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matured from the transfected and untransfected male stem cells is relatively low. To overcome this problem, we are going to test the possibility of selecting sperm with exogenous DNA from ejaculated sperm by flow cytometry using an antibody, and we will attempt to improve the efficiency of transgenic livestock production by in vitro fertilization with concentrated sperm harboring foreign DNA. After the completion of this manuscript, we have critical evidence to verify our data (Ogawa et al., 1995), indicating that spermatozoa derived from transfected germ cells can transfer exogenous DNA into oocytes.

ACKNOWLEDGMENTS

The authors thank Dr. Karan Artzt (University of Texas at Austin) for the gift of the Celsep® machine. We thank H.C. Kim (Hanyang University) for the histological preparation of the slides and the immunohistochemistry. We also thank Dr. Prof. P. Tryhun and Dr. D. Veron Reyner (Rowett Research Institute, UK) for critical reading of the revised manuscript. This study was supported in part by Korean Agriculture Special Tax Grants.

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